VOL. 57 NO. 3, MAR. 2004 THE JOURNAL OF ANTIBIOTICS pp. 197 — 204

CE-108, a New Macrolide Tetraene Antibiotic

Francisco J. Pérez-Zúñiga[a], Elena M. Seco[a], T. Cuesta[a], Falko Degenhardt[b], Jürgen Rohr[b], Carlos Vallín[c], Yanelis Iznaga[c], Marta E. Pérez[c], Leonora González[c] and Francisco Malpartida[a,*]

[a] Centro Nacional de Biotecnología, Campus de la UAM, 28049 Cantoblanco, Madrid, Spain
[b] College of Pharmacy, University of Kentucky, 907 Rose Street, Lexington, KY 40536-0082, USA
[c] Centro de Química Farmaceútica, Calle 200 y 21, Atabey, Cubanacan, La Havana, Cuba

(Received for publication October 10, 2003)

In the search for strains producing antifungal compounds, a new tetraene macrolide CE-108 (**3**) has been isolated from culture broth of *Streptomyces diastaticus* 108. In addition, the strain also produces the previously described tetraene rimocidin (**1**) and also the aromatic polyketide oxytetracycline. Both tetraene compounds, structurally related, are produced in a ratio between 25 to 35% (CE-108 compared to rimocidin), although it can be inverted toward CE-108 production by changing the composition of the fermentation medium. This paper deals with the characterization of the producer strain, fermentation, purification, structure determination and biological properties of the new macrolide tetraene CE-108.

The needs for antifungal compounds have increased considerably in the last decades. The growing number of high-risk patients such as immunocompromised or treated with immunosupressors, led to a strong proliferation of fungal systemic infections on these populations. Traditionally, the polyene macrolide and azole-derived compounds played a crucial role in the treatment of those infections. The extensive use of the azole-derived antimycotics had led to the appearance of strains resistant to these compounds[1]; in contrast, amphotericin B, which undoubtedly is the leading antifungal drug even after several decades of clinical use, have strong toxicity[2]. More recently a newer class of antifungal agents, inhibitors of the (1,3)-β-D-glucan synthase (echinocandins), have been described as promising new antifungal drugs[3]. Thus, despite improved formulations of amphotericin B[4] or newer chemical variants of azole compounds[5], there are needs for new antifungal agents in order to fight the growing mycotic diseases.

Several approaches are already in progress for isolation of new antifungal compounds: new target selection, rational chemical modification of existing leads, combinatorial biosynthesis, genetic modification of producer strains, *etc.* This paper is dealing with the characterization of a new tetraene (CE-108) by a newly isolated strain (*Streptomyces diastaticus* var. 108) which also produces the previously known rimocidin. We firmly believe that the new molecule will increase the chemical bank of natural products and thus providing more elements for further manipulation of existing molecules.

Results

Taxonomy of *Streptomyces diastaticus* var. 108

The vegetative mycelium of 108 strain grew moderately on both synthetic and complex media and did not show fragmentation into coccoid or bacillary elements. The spore chains were “retinaculum apertum” type and the surface of the spores was smooth. No sclerotic granules, sporangium or zoospore were observed.

The physiological properties and cultural characteristics of the producer strain are shown in Tables 1 and 2 respectively. The vegetative mycelium showed yellowish

* Corresponding author: fmalpart@cnb.uam.es

Table 1. Physiological characteristics of *Streptomyces diastaticus* 108.

Condition	Characteristics	Condition	Characteristics
Growth in the presence of (% w/v)		Melanin formation on tyrosine agar	-
Phenol (0.1)	-	**Growth on**	
Sodium azide (0.01)	+	Peptone-Yeast extract-iron agar	-
Sodium chloride (7.0)	+	Tyrosine-yeast extract broth	-
Growth temperatures 45ºC	+	Starch hydrolysis	-
Carbohydrate utilization		Milk coagulation	+
D-glucose	+	Milk peptonization	+
L-rabinose	-	Nitrate reduction	+
D-xylosew	-	H_2S production	-
D-fructose	+	**Degradation activity**	
Sucrose	-	Pectin	-
L-Rhamnose	-	Arbutin	+
Raffinose	-	Allantoin	-
Inositol	+	Xanthine	-
D-Manitol	+	**Use of nitrogen source**	
Enzymatic Activity		L-asparagine	+
Lecithinase	+	a-aminobutiric acid	+
Antimicrobial activity		Histidine	+
Aspergillus niger	+	hidroxyproline	-
Bacillus subtilis	+	**Resistance to antibiotics (μ/ml)**	
Streptomyces neurinus	+	Neomycin (100)	+
		Rifampicin (50)	+
		Hygromycin B (200)	+
		Kanamycin (200)	
		Apramycin (200)	
		Gentamicin (100)	

(+) Yes
(-) No

brown colour on various media. The aerial mycelium showed grey-white to reddish colours. Based on the observed taxonomic properties the producer strain is thought to belong to the genus *Streptomyces* and close to *Streptomyces diastaticus*; thus, the strain is named as *Streptomyces* diastaticus var. 108.

Characterization of the Active Secondary Metabolites being Produced by *S. diastaticus* var. 108

S. diastaticus var. 108 was cultivated in SYM2 medium. After 72 hours the biological activity of the culture broths were tested for antibiotic activity against *Micrococcus luteus* and *Penicillium chrysogenum*. The culture showed biocide activity against both strains indicating the presence of one or more antibacterial and antifungal compounds. In order to know if both biological activities were due to the same or different compounds, the fermentation broth was fractionated. Thus, cells from 4 days old cultures were removed by centrifugation at 5,000 *g*; the supernatant was freeze dried and stored until further used. Aliquots of the lyophilized material were reconstituted in water, at the same concentration as the original cultures, filtered through 0.45 μm and slowly loaded (10 ml) on a semi-preparative Amberlite XAD16 column (150×10 mm). The column was washed with ten volumes of 20% methanol in water and the bound substances eluted using a linear gradient methanol; the gradient was controlled with a Water Automated Gradient Controller using the profile shown on Table 3.

Fractions were collected at 3 minutes intervals and one hundred microliters of each were assayed for its biological activity against *Micrococcus luteus* and

Table 2. Cultural characteristics of *Streptomyces diastaticus* 108.

Medium	Growth	Reverse side colour	Aerial mycelium	Soluble pigment
Yeast extract-broth triptone (ISP-1)	Moderate	none	none	none
Yeast extract malt extract agar (ISP-2)	Moderate	yellowish brown	grey white	none
Oatmeal agar (ISP-3)	Moderate	yellowish brown	grey white	none
Inorganic salts-starch agar (ISP-4)	Bad	yellowish brown	yellowish redwish	none
Glycerol-asparagine agar (ISP-5)	Moderate	Yellowish grey	none	none
Peptone-Yeast extract-iron agar (ISP-6)	Bad	yellowish	none	none
Tyrosine agar (ISP-7)	Bad	white	none	none
Nutrient agar	Bad	yellowish brown	Grey white	none
SYM	Moderate	yellowish brown	Grey-redwish	none

Table 3. Gradient table for semi-preparative fractionation.

Time (min)	Flow (ml/min)	% Phase A (methanol)	% Phase B (water)	Curve
0	1.0	20	80	-
65	1.0	20	80	6
80	1.0	100	0	11

Table 4. Gradient table for analytical fractionation.

Time (min)	Flow (ml/min)	%A	%B	Curve
	0.6	0.0	100.0	
5.0	0.6	0.0	100.0	6
10.0	0.6	50.0	50.0	6
20.0	0.6	100.0	0.0	9
25.0	0.6	100.0	0.0	9
30.0	0.6	0.0	100.0	11

A: Mobile Phase: MetOH
B: Mobile Phase: NH_4-Acet 20 mM pH 5, 20% EtOH

Penicillium chrysogenum. The distribution of both activities through the gradient fractions (see Figure 1) showed that antibacterial and antifungal activities could can be separated suggesting that both activity might be due to different compounds.

The fractions showing antibacterial or antifungal activities were pooled separately (without mixing activities) and subjected to further analysis by HPLC. Thus, small samples from each set were resolved using a Kromasil C8 analytical column (100×4.6 mm) with the gradient profile shown on Table 4.

The fractions carrying antibacterial activity showed a major compound with an UV spectrum different to that of the fractions carrying the antifungal activity. The spectrum of the main compound present in these fractions, matched perfectly with that of tetracycline-type molecules. Samples carrying antifungal activity showed two main components whose UV spectra correlates well with that of polyene compounds of the tetraene group. The chromatographic properties of the antibacterial compound are indistinguishable of those of oxytetracycline: the retention time is similar to a commercial oxytetracycline sample when analyzed under different chromatographic conditions; thus, the antibacterial compound is likely oxytetracycline. No further work was carried out with this activity.

Fig. 1. Fractionation of antimicrobial activities of *S. diastaticus* var. 108 fermentation broth.

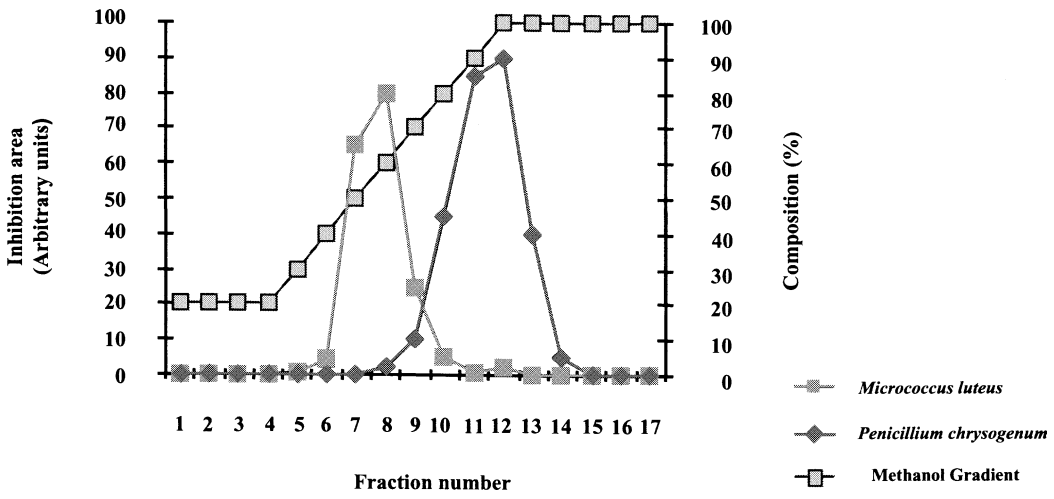


Table 5. Minimal inhibitory concentration of CE-108 and rimocidin MIC (μg/ml).

Organism	CE-108 (#1)	Rimocidin (#2)
Candida albicans	32	16
Candida krusei	32	16
Cryptococcus neoformans	16	8
Aspergillus niger	8	8
Aspergillus fumigatus	8	8
Aspergillus candidus	8	8
Fusarium oxysporum	8	8
Microsporum canis	16	8
Microscporum gypseum	8	4
Trichophyton mentagrophytes	8	4
Trichophytum rubrum	16	8
Trichophyton tonsurans	16	8

Isolation and Structural Elucidation of the Produced Polyenes

For elucidation of the chemical structures of the polyenes, both compounds were separately purified. *S. diastaticus* var. 108 was grown on R5 solid medium[6] at 28°C. Solid pieces were used to inoculate a liquid soybean meal/mannitol (2%/2%) medium, in which the strain was grown for 3 days. The culture filtrate was lyophilized, and then chromatographed on RP-18 silica gel (UV-detection at 304 nm) to yield one major and one minor compound.

The positive FAB mass spectrum of the major compound revealed a mass of *m/z* 768 [M−H$^+$], which along with the NMR spectra allowed the deduction of a molecular formula of $C_{39}H_{61}NO_{14}$. This molecular formula as well as the melting point, the UV and the NMR data revealed that this compound was most likely identical with the literature-known macropolyene antibiotic rimocidin (**1**). Final proof of its identity with rimocidin including the so far known stereochemistry came from a better set of NMR data obtained after its chemical conversion into a better soluble derivative, which was obtained in a two step procedure, (i) acetylation of the amine function of its mycosamine moiety, and (ii) amidation of the carboxylic acid function with glycine methyl ester. This derivative (**2**) could be directly compared to the same derivative obtained from an authentic sample of rimocidin[7]. With the exception of one signal in the ^{13}C-NMR (we observed the C-3′ signal at δ_C 56.2, which is typical for an *N*-substituted carbon, however, it was listed at $\delta_{C\text{-}3'}$ 68.3 in the literature[7] all NMR data

Table 6. ^{1}H-NMR-data (500 MHz, d_4-methanol) of CE-108; ^{13}C-NMR-data (125.7 MHz, d_4-methanol).

Position	^{1}H δ (ppm)	Multiplicity (*J*/Hz)	^{13}C δ (ppm)
1			173.4
2	2.18	1H, dt (9.6, 3.8)	56.3
2''	1.59;1.90	2H, dq (7.5, 3.8)	22.4
2''-Me	0.92	3H, t, (7.5)	10.9
3	4.11	1H, dd (9.6, 2.2)	68.5
4	2.37;2.46	2H, m	48.8
5			210.6
6	2.46;2.32	2H, m	44.3
7	1.39;1.61	2H, m	19.6
8	1.39;1.30-1.26	2H, m	37.6
9	4.12	1H, ddt (9.3, 8.2, 2.2)	68.4
10	1.61	2H, m	46.5
11			97.8
12	2.02;1.30-1.26	2H, m	44.1
13	4.29	1H, ddd (11.1, 10.5, 4.6)	66.9
14	2.03	1H, t (9.3)	60.5
-COOH			179.3
15	4.37	1H, dd (9.3, 8.2)	66.6
16	2.30;1.70	2H, dd (8.2)	38.7
17	4.45	1H, td (3.5, 8.3)	78.2
18	5.95	1H, dd (15.3,8.3)	136.0
19	6.35	1H, dd (13.8,10.7)	133.6
20	6.13	1H, m	129.3*
21	6.13	1H, m	133.4*
22	6.13	1H, m	132.3*
23	6.13	1H, m	132.3*
24	6.13	1H, m	132.6*
25	5.63	1H, ddd (14.0, 9.5, 5.1)	131.1
26	2.32; 2.40	2H, m	40.0
27	5.07	1H, ddq (10.5, 6.3, 1.9)	70.8
28	1.26	3H, d (6.3)	20.0
Sugar			
1'	4.62	1H, s	98.2
2'	4.03	1H, d (2.8)	68.0
3'	3.20	1H, dd (9.0, 2.8)	56.1
4'	3.37[a]	1H, m	69.5
5'	3.35[a]	1H, m	73.5
6'	1.31	3H, d (5.7)	16.7

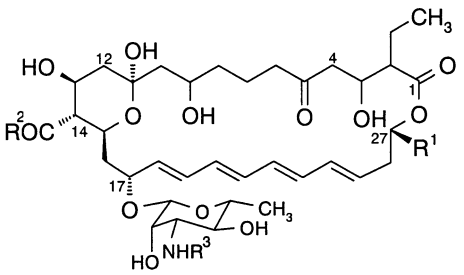
[a] Signals are partially obscured by solvent; * assignments interchangeable

were identical. Since the optical rotation of this major polyene compound also matched the reported value for rimocidin,[8,9] there was no doubt that the main polyene of *Streptomyces diastaticus* var. 108 and rimocidin were identical compounds (and that there was a wrong chemical shift given[7] for C-3′).

The molecular formula of the minor polyene was deduced as $C_{37}H_{57}NO_{14}$ in agreement with the positive-HR-FAB mass spectrum and the NMR data. The NMR data also revealed that this compound is a novel close relative of rimocidin. The comparison of the NMR data of its derivative **4** (obtained in the same way as described above for rimocidin), revealed that both compounds are identical with the exception of the alkyl side chain attached at C-27, which is a methyl group here instead of the propyl group found in rimocidin (Table 6). The connection of this methyl side chain (C-28) to C-27 was proven by a $^{3}J_{C\text{-}H}$ long-range coupling, observed in the HMBC-spectrum between 28-H_3 and C-26. The coupling constants found in the ^{1}H-NMR spectrum of derivative **4**, which are identical with those of the analogous rimocidin derivative **2** and a nearly identical $[\alpha]_D$ value combined with the fact that CE-108 is a biosynthetic starter-chain variant of rimocidin suggests that all stereochemical centers are identical to those in rimocidin. In summary the minor polyene compound from *Streptomyces diastaticus* var. 108, now named CE-108, has the structural formula **3**.

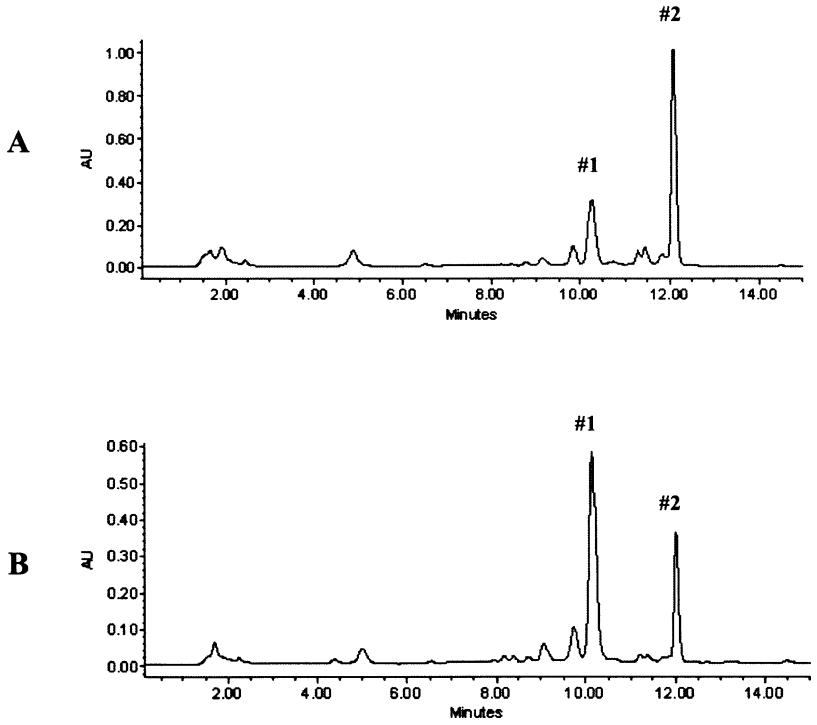
Physicochemical Data of CE-108 (**3**)

$[\alpha]_D^{25} = +117°$ (c=1, pyridine); MW: 739.77 g/mol. ($C_{37}H_{57}NO_{14}$); FAB-MS: positive: *m/z* 740 ($[M+H]^+$; HR: calculated for $C_{37}H_{58}NO_{14}$ 740.3857, found; 740.3862); negative: *m/z* 738 ($[M-H]^-$); UV (Methanol) λ_{max} [nm] (ε): 317 (54700); 302 (60300); 290 (44200). NMR data: see Table 6.



1 $R^1 = CH_3CH_2CH_2$; R^2 = OH; R^3 = H
2 $R^1 = CH_3CH_2CH_2$; $R^2 = NHCH_2COOCH_3$; $R^3 = COCH_3$
3 $R^1 = CH_3$; R^2 = OH; R^3 = H
4 $R^1 = CH_3$; $R^2 = NHCH_2COOCH_3$; $R^3 = COCH_3$

Fig. 2. HPLC analysis of methanolic extracts from cultures of *S. diastaticus* var. 108.



Supernatants from: A, SYM2 medium; B: R5 medium. #1: CE-108; #2. rimocidin.

Biological Activity of the Polyenes Produced by *S. diastaticus* var. 108

Similarly to rimocidin, the tetraene CE-108 showed no antibacterial activity; the antifungal activity of the produced tetraenes was tested against several strains; the corresponding MICs are given in Table 5.

Cytotoxicity Activity

The cytotoxicity experiments carried out with CE-108 and rimocidin gave an IR_{50} of 130 and 64 μg/ml respectively. These results indicated that CE-108 is less toxic than rimocidin.

Production of Polyenes

In order to test how both polyenes are being produced, the producer strain was cultivated in a variety of different liquid media (SYM2, R5, TSB, GAE, DNA, YEA and AHK). All these media (50 ml in 500 ml flask) were inoculated with 1 ml of a 48 hours old culture grown on SYM2. After 72 hours, aliquots from each culture were extracted with methanol as indicated in Material and Methods, and the methanolic extracts analyzed by HPLC under analytical conditions. The results showed that the maximal production of polyenes is taking place in SYM2 and R5 media. It is noteworthy that in SYM2 medium the production of rimocidin is three to four times that of CE-108, while in R5 medium the production profile is inverted toward CE-108 (see Figure 2).

Discussion

The strain characterized in this work (*S. diastaticus* var. 108) resembles many other Actinomycetes, in the sense that it produces several bioactive compounds. Similarly to our strain, *Streptomyces rimosus* is known to produce oxytetracycline and rimocidin[8,10], but it is not reported to produce the congener CE-108. This metabolic diversity in a single strain is not an exception; *S. coelicolor*, known to produce several antibiotics (actinorhodin, prodigiosin, methylenomycin and CDA), with the genome

fully annotated, it is expected to produce several more secondary metabolites[11]; similarly, *Streptomyces avermitilis*, well known as avermectin producer, carries several biosynthetic clusters for production of some other secondary metabolites[12]. This ability (or at least to have the genetic capability) to produce several compounds is, at least potentially, an attractive tool toward the identification of new bioactive molecules. At least conceptually, it became an interesting possibility to pay attention to those related compounds, which might well be present in small quantities within the fermentation broth of producer strains. This had proved in the past to be a valuable tool not only for understanding the biochemistry of several biosynthetic pathways, but also for finding out new variants of interesting pharmaceuticals; in some cases these molecules can be directly exploited as led compounds or their pathways, once the genes became available, used for modification of other interesting compounds by meaning of combinatorial biosynthesis. The structure (**3**) of CE-108 is also no surprise, since it is a polyketide starter unit variant of the major compound rimocidin (**1**). It has been shown several times that the loading modules in Type I polyketide synthases (Type I-PKS) are flexible regarding the starter unit[13,14]. Here, acetate has been utilized for the production of **3** instead of the usual butyrate starter used for the rimocidin biosynthesis.

General Experimental Procedures

Screening for Biological Activities

The producer strain *S. diastaticus* 108 was isolated from a soil sample collected in the Holguin province in Cuba (Nature Reserve in Moa). Polyene susceptibility assay method described by ETIENNE *et al.*[15] was used. Twenty-five ml of Sabouraud Dextrose agar[16] were inoculated with 0.4% (v/v) of a microorganism suspension (yeast or filamentous fungus). Tween 80, dissolved in 95% ethanol, was added aseptically to the molten agar medium at a final concentration of 4%. Antimicrobial activities were tested by the disk assay method. Known macrolide polyenes (amphotericin B, nystatin and pimaricin) were used as positive controls.

Taxonomic Studies

The morphological properties of the producer were observed with a scanning electron microscope (Jeol Model JEM-100S). The cultural characteristics and physiological properties were determined by the methods of PRIDHAM and GOTTLIEB[17] with the media recommended by WAKSMAN[18] and SHIRLING and GOTTLIEB[19]. The identification was carried out according to the procedures of WILLIAMS *et al.*[20].

Metabolites Extraction and Analysis

The fermentation products were analyzed by high performance liquid chromatography (Water 626 Pump) equipped with a Photodiode Array Detector (PDA) 996 and computer controlled by Millennium 2010 Chromatography Manager. For routine assays, samples containing both, growing mycelia and fermentation broths, were adjusted to pH 3 with formic acid and extracted with 20 volumes of methanol; the precipitated material was removed by centrifugation at 13,000 r.p.m. (Heraeus, Biofuge 13) and the supernatant applied directly to a Kromasil C8 column (4.6×100 mm). The samples were fractionated for analytical conditions by a binary gradient (Table 4); the chromatograms were monitored by setting the wavelength to 304 nm; for large-scale preparation the compounds were separated using the gradient shown on Table 3 and 150×30 mm column packaged with C8 silica resin.

Media and Fermentation Conditions

The media used for batch cultures, unless otherwise indicated, were: SYM2: 2% meat extract, 0.4% triptone, 0.5% soy peptone, 0.2% glucose, 2.5% soluble starch, 0.3% yeast extract, pH7.0; R5[6]; TSB[6]; GAE[16], YEA[16]; AHK: 3% soluble starch, 2% soybean meal, 0.01% K_2HPO_4, 0.01% $FeSO_4$; DNA[6]. For testing purposes, the strain was cultivated in 250 ml flask at 28°C in a reciprocal rotary shaker at 300 r.p.m.

Large scale fermentations were performed in a Braun MD 2 liters fermentor containing 1.5 liters of SYM2 medium and carried out at 28°C. The Oxygen was adjusted to >50% by adjusting both, agitation (between 400 to 800 rpm) and air flow (between 1 to 8 liters/minute. Inocula were prepared by growing the strain for two days in 500 ml flask with 50 ml of SYM2 medium; the flasks previously inoculated with 10^8 spores were grown for 48 hours before inoculation of the MD fermentors.

MIC Determination

Susceptibility of the tested microorganism to the polyenes was determined using the agar dilution method as described by WASHINGTON[21].

Cytotoxicity Assays

Vero cell lines (epithelial cells of green monkey kidney) were cultivated in the RPMI-1640 medium supplemented with 10% foetal bovine serum at 37°C in a CO_2 incubator. The cells were treated with various concentrations of both

CE-108 and rimocidin, purified from culture broths of the producer strain and dissolved in DMSO. After 72 hours in the presence of the drugs, the cells were taken and the IC_{50} values were determined by counting viable cells using the microculture terazolium assay (MTT assay)[22].

Acknowledgements

This work was supported by Grants to F.M from Spanish CICYT (BIO1999-1223), EU (QLRT-2000-00131), to F.M. and C.V.: Consejo Superior de Investigaciones Científicas/ Agencia de Ciencia y Tecnología para el Desarrollo de Cuba and to C.V. from Cuban CITMA 00402025.

References

1) Rex, J. H.; M. G. Rinaldi & M. A. Pfaller: Resistance of *Candida* species to fluconazole. Antimicrob. Agents Chemother. 39: 1～8, 1995
2) Gallis, H. A.; R. H. Drew & W. W. Pickard: Amphotericin B: 30 years of clinical experience. Rev. Infect. Dis. 12: 308～329, 1990
3) Onishi, J.; M. Meinz, J. Thompson, J. Curotto, S. Dreikorn, M. Rosenbach, C. Douglas, G. Abruzzo, A. Flattery, L. Kong, A. Cabello, F. Vicente, F. Pelaez, M. T. Diez, I. Martin, G. Bills, R. Giacobbe, A. Dombrowski, R. Schwartz, S. Morris, G. Harris, A. Tsipouras, K. Wilson & M. B. Kurtz: Discovery of novel antifungal (1,3)-beta-D-glucan synthase inhibitors. Antimicrob. Agents Chemother. 44: 368～377, 2000
4) Hiemenz, J. W. & T. J. Walsh: Lipid formulations of amphotericin B: recent progress and future directions. Clin. Infect. Dis. 22 (Suppl. 2): S133～S144, 1996
5) Patterson, T. F.: Role of newer azoles in surgical patients. J. Chemother. 11: 504～512, 1999
6) Kieser, T.; M. J. Bibb, M. J. Buttner, K. F. Chater & D. A. Hopwood: Practical *Streptomyces* Genetics. Norwich, 2000
7) Sowinski, P.; J. Pawlak & E. Borowski: Stereostructure of rimocidin. J. Antibiotics 48: 1288～1291, 2003
8) Davisson, J. W.; F. W. Tanner & A. C. Finlay: Rimocidin, a new antibiotic. Antibiot. Chemother. 1: 289～290, 1951
9) Pandey, R. C. & K. L. Rinehart, Jr.: Polyene antibiotics. VII. Carbon-13 nuclear magnetic resonance evidence for cyclic hemiketals in the polyene antibiotics amphotericin B, nystatin A1, tetrin A, tetrin B, lucensomycin, and pimaricin. J. Antibiotics 29: 1035～1042, 1976
10) Behal, V. & I. S. Hunter: Tetracyclines. *In* Genetics and Biochemistry of Antibiotic Production. *Ed.* L. C. Vining & F. W. Tanner, pp. 359～385. Butterworth-Heinemann, Boston, Massachusetts, 1995
11) Bentley, S. D.; K. F. Chater, A. M. Cerdeno-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabbinowitsch, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill & D. A. Hopwood: Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417: 141～147, 2002
12) Omura, S.; H. Ikeda, J. Ishikawa, A. Hanamoto, C. Takahashi, M. Shinose, Y. Takahashi, H. Horikawa, H. Nakazawa, T. Osonoe, H. Kikuchi, T. Shiba, Y. Sakaki & M. Hattori: Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. Proc. Natl. Acad. Sci. USA 98: 12215～12220, 2001
13) Marsden, A. F.; B. Wilkinson, J. Cortes, N. J. Dunster, J. Staunton & P. F. Leadlay: Engineering broader specificity into an antibiotic-producing polyketide synthase. Science 279: 199～202, 1998
14) Pacey, M. S.; J. P. Dirlam, R. W. Geldart, P. F. Leadlay, H. A. Mcarthur, E. L. Mccormick, R. A. Monday, T. N. O'Connell, J. Staunton & T. J. Winchester: Novel erythromycins from a recombinant *Saccharopolyspora erythraea* strain NRRL 2338 pIG1. I. Fermentation, isolation and biological activity. J. Antibiotics 51: 1029～1034, 1998
15) Etienne, G.; E. Armau & G. Tiraby: A screening method for antifungal substances using *Saccharomyces cerevisiae* strains resistant to polyene macrolides. J. Antibiotics 43: 199～206, 1990
16) Atlas, R. M.: Microbiological Media. CRC Press, Boca Raton, Florida, 1993
17) Pridham, T. G. & D. Gottlieb: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107～114, 1948
18) Waksman, S. A.: Classification, identification and description of genera and species. *In* The Actinomycetes. -VII-363.Williams and Wilkins Co, Baltimore, 1961
19) Shirling, E. B. & D. Gottlieb: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313～340, 1966
20) Williams, S. T.; M. Goodfellow, E. M. Wellington, J. C. Vickers, G. Alderson, P. H. Sneath, M. J. Sackin & A. M. Mortimer: A probability matrix for identification of some Streptomycetes. J. Gen. Microbiol. 129: 1815～1830, 1983
21) Washington, J. A.: Susceptibility test: agar dilution. *In* Manual of Clinical Microbiology. *Eds*. E. H. Lennette *et al.* 967～971. American Society for Microbiology, Washington D.C., 1985
22) Mosmann, T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65: 55～63, 1983